Purification and Characterization of Hydrolase with Chitinase and Chitosanase Activity from Commercial Stem Bromelain

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A hydrolase with chitinase and chitosanase activity was purified from commercial stem bromelain through sequential steps of SP-Sepharose ion-exchange adsorption, HiLoad Superdex 75 gel filtration, HiLoad Q Sepharose ion-exchange chromatography, and Superdex 75 HR gel filtration. The purified hydrolase was homogeneous, as examined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The enzyme exhibited chitinase activity for hydrolysis of glycol chitin and 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside [4-MU-β-(GlcNAc)$_3$] and chitosanase activity for chitosan hydrolysis. For glycol chitin hydrolysis, the enzyme had an optimal pH of 4, an optimal temperature of 60 °C, and a K_m of 0.2 mg/mL. For the 4-MU-β-(GlcNAc)$_3$ hydrolysis, the enzyme had an optimal pH of 4 and an optimal temperature of 50 °C. For the chitosan hydrolysis, the enzyme had an optimal pH of 3, an optimal temperature of 50 °C, and a K_m of 0.88 mg/mL. For hydrolysis of chitosans with various N-acetyl contents, the enzyme degraded 30−80% deacetylated chitosan most effectively. The enzyme split chitin or chitosan in an endo-manner. The molecular mass of the enzyme estimated by gel filtration was 31.4 kDa, and the isoelectric point estimated by isoelectric focusing electrophoresis was 5.9. Heavy metal ions of Hg^{2+} and Ag^+, p-hydroxymercuribenzoic acid, and N-bromosuccinimide significantly inhibited the enzyme activity.

KEYWORDS: Chitinase/chitosanase; purification; characterization; stem bromelain

INTRODUCTION

Chitosan can be defined as partially or fully deacetylated chitin containing $(1-4)$-β-D-glucosamine residues (*1*). It occurs in the cell walls of all *Zygomycete* fungi (*1*–*4*) and may occur in small amounts in the cell walls of other fungi (*5*, *6*) and also in certain green algae such as chlorella (*7*). It is also produced commercially by alkaline deacetylation of shellfish (usually crab) chitin. Chitosan and chitosan oligosaccharides are useful in the food (*8*–*10*), agriculture (*11*–*15*), and medicine industries (*16*–*19*). For example, chitosan can activate the defense response of plants. It can also directly inhibit the growth of a wide range of microorganisms. Chitooligosaccharides and N-acetylchitooligosaccharides have recently attracted much attention for their various physiological activities including antifungal activity (*10*, *13*, *14*) and immune-enhancing activity (*16*–*19*). Chitosanases (EC 3.2.1.132) are hydrolases acting on chitosan. They are found in bacteria, fungi, and plants (*20*). They are generally endo-splitting enzymes and could hydolyze chitosan into chitooligosaccharides and glucosamine. The distinction between chitosanase and chitinase is not sharp, as both enzymes have the ability to degrade a variety of chitosans with different degrees of acetylation (*21*–*23*). However, chitosanases are distinguished by their lower apparent mass (*24*) and substrate specificity. Chitosanase act preferentially on highly deacetylated chitosan. Up to now, chitosanase activity has been studied mostly in bacteria (*25*–*27*) and fungi (*28*, *29*), although several molecular forms of plant chitosanase have been found in cucumbers (*30*) and a few other plant species (*31*). However, chitosanase has not yet been thoroughly purified and characterized from plant origins. Plant chitinase and β-1,3-glucanases have long been suggested to belong to the antifungal defenses of plants because they inhibit fungal growth in model experiments (*32*, *33*). It is believed that chitinase and β-1,3-glucanase act as defense proteins in higher plants, protecting the plant from fungal pathogens. As chitinase and β-1,3-glucanases from plants, chitosanase or chitinase with chitosanase activity has received attention because of its potential lytic activity against the fungal walls of pathogens. (*25*, *26*, *34*) and potenial use for preparation of low molecular weight chitosan or chitooligosaccharides (*35*, *36*).

Recently some commercial crude enzyme preparations from plant origins such as papain, stem bromelain, ficin, and wheat germ lipase (*36*–*38*) displayed lytic activities toward chitosan. Terbojevich et al. (*39*) reported that chitin-immobilized crude

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papain could be used for continuous hydrolysis of chitosan for the preparation of chitosan hydrolysates with different molecular weights. Yalpani and Pantaleone (*36*) found that crude stem bromelain and pepsin were more efficient catalysts for chitosan hydrolysis than both a commercial crude chitinase and a lysozyme preparation. However, the lytic enzymes involved in chitosan hydrolysis in these crude enzyme preparations heve not yet been confirmed. To demonstrate the presence of chitosanolytic enzyme in commercial stem bromelain and to determine its biochemical properties, we purified and characterized the chitosanolytic enzyme from stem bromelain preparation. We found that this chitosanolytic enzyme also showed chitinolytic activity. In this paper, we describe the purification and some properties of this hydrolase.

MATERIALS AND METHODS

Chemicals. 4-Methylumbelliferyll β-D-*N*,*N*′*N*′′-triacetylchitotrioside [4-MU-β-(GlcNAc)$_3$], *N*-acetyl-D-glucosamine, glucosamine, 4-methylumbelliferone, ethyl acetimidate (EAM), *p*-hydroxymercuribenzoate (PHMB), diethyl pyrocarbonate (DEPC), *N*-ethyl-5-phenylisoxazoline-3′-sulfonate (Woodward's reagent K; WRK), phenylmethanesulfonyl fluoride (PMSF), 1,2-cyclohexanedione (CHD), 2,4-dinitro-1-fluorobenzene (DNFB), and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO). Crab chitin and chitosan (82% deacetylation) were obtained from Ohka Enterprises Co. Ltd. (Kaohsiung, Taiwan). SP Sepharose Fast Flow, HiLoad Superdex 75 16/60 HR column, HiLoad Q Sepharose Fast Flow 16/10 HR column, Superdex 75 10/30 HR column, PhastGel IEF 3-9, and broad p*I* kit (p*I* = 3.5–9.3) were from Pharmacia (Uppsala, Sweden). Stem bromelain (catalog no. 101651) and *N*-bromosuccinimide (NBS) were from Merck (Darmstadt, Germany). Bicinchoninic acid protein assay reagent was obtained from Pierce (Rockford, IL). Glucosamine oligomers [(GlcN)$_{2-6}$] and *N*-acetylglucosamine oligomers [(GlcNAc)$_{2-5}$] were from Seikagaku Corp. (Tokyo, Japan). Potassium polyvinyl sulfate solution (N/400) was purchased from Wako (Osaka, Japan). Buffer salts and other chemicals used were of reagent grade.

Chitinase/Chitosanase Purification. Unless otherwise stated, all purification steps were carried at 4 °C. One gram of commercial stem bromelain was dissolved in 25 mL of 50 mM phosphate buffer (sodium salt, pH 7.2) and stirred for 20 min. The precipitate was removed by centrifugation at 15000*g* for 10 min. Five milliliters of SP Sepharose Fast Flow cation exchanger was equilibrated with 50 mM posphate buffer (sodium salt, pH 7.2) and then added to the supernatant for adsorption of bromelain enzyme (isoelectric point of stem bromelin is 9.55). The mixture was shaken for 30 min. After centrifugation at 15000*g* for 10 min, the supernatant was collected. The procedure was repeated once again with 5 mL of the cation exchanger. The supernatant was then dialyzed with 25 mM imidazole–HCl buffer (pH 7.4). After lyophilization, the enzyme was dissolved in 1 mL of 25 mM imidazole–HCl buffer (pH 7.4) and applied to a HiLoad Superdex 75 (16/60) column. The column was eluted with 20 mM imidazole–HCl buffer (pH 7.4) using an FPLC system (Pharmacia) at 20 °C at a flow rate of 30 mL/h. Fractions (2 mL/fraction) containing the enzyme activity were collected and dialyzed with 20 mM Tris-HCl buffer (pH 8.0). After lyophilization, the enzyme was dissolved in 2 mL of 20 mM Tris-HCl buffer (pH 8.0) and applied to a HiLoad Q Sepharose (16/10) column. The column was washed with 20 mL of 20 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of NaCl from 0 to 0.5 M in an equilibrium buffer using an FPLC system (Pharmacia) at 20 °C at a flow rate of 30 mL/h. Fractions (1 mL/fraction) containing the enzyme activity were collected. After lyophilization, the enzyme was dissolved in 2 mL of 25 mM imidazole–HCl buffer (pH 7.4) and applied to a Superdex 75 HR column (10/30). The column was eluted with 25 mM imidazole–HCl buffer (pH 7.4, 0.1 M NaCl) using an FPLC system (Pharmacia) at 20 °C at a flow rate of 12 mL/h. Fractions (0.5 mL/fraction) containing the enzyme activity were collected.

Glycol Chitin Synthesis. Glycol chitin was obtained from the chitin reaction with ethylene chlorohydrin (2-chloroethanol) under alkaline conditions according to the method (procedure B) described by Hirano

(*40*). Powdered chitin (4 g) was slurried with a 60% (w/w) sodium hydroxide solution (16 mL), containing 0.2% sodium dodecyl sulfate, at 4 °C for 1 h, and then the slurry was kept in a freezer at −20 °C overnight. After thawing and repeat keeping in a freezer at −20 °C overnight, the slurry of frozen alkali chitin obtained was added, in portions, to 2-propanol (70 mL), containing ethylene chlorohydrin (16 mL), in an ice bath over 30 min. The mixture was mechanically stirred at room temperature for 1 h, and the product was collected by filtration through a glass filter paper and washed well with ethanol to give a powdered material. The product was N-acetylated by the addition of acetic anhydride, neutralized with acetic acid, dialyzed with water, and lyophilized to give salt-free glycol chitin.

Chitinase Activity Determination. Chitinase activity was determined using 4-MU-β-(GlcNAc)$_3$ or glycol chitin as a substrate.

(A) 4-MU-β-(GlcNAc)$_3$ as Substrate. The incubation mixture contained 50 μL of 50 μM 4-MU-β-(GlcNAc)$_3$, 500 μL of 0.05 M acetate buffer (pH 4.0), and 450 μL of diluted enzyme solution (0.001–0.002 fluorometric unit) in a total volume of 1 mL. The incubation was carried out at 50 °C for 1 h. One milliliter of 1 M glycine/NaOH buffer (pH 10.6) was added to enhance the fluorescence. The amount of fluorescence was measured at 355 nm excitation and 460 nm emission using a Hitachi fluorescence spectrophotometer (model F-4500) (*41*). At least two measurements were made and the results averaged. One unit of enzyme activity for 4-MU-β-(GlcNAc)$_3$ hydrolysis was defined as the amount of enzyme releasing 1 nmol of 4-methylumbelliferone (MU) per minute under assay conditions.

(B) Glycol Chitin as Substrate. The reaction mixture contained 0.9 mL of 0.1 M acetate buffer (pH 4.0), 1 mL of 0.2% glycol chitin, and 0.1 mL of diluted enzyme solution (0.005–0.01 colorimetric unit) in a total volume of 2 mL. The incubation was carried out at 45 °C for 30 min. The reducing sugar produced was measured colorimetrically (decrease of absorbance at 405 nm) with a ferri-ferrocyanide reagent using the method described by Imoto and Yagishita (*42*). At least two measurements were made and the results averaged. One unit of enzyme activity for glycol chitin hydrolysis was defined as the amount of enzyme releasing 1 μmol of *N*-actylglucosamine per minute under assay conditions.

Chitosanase Activity Determination. Chitosanase activity was determined using a viscosimetric or colorimetric method using a chitosan acetate solution.

(A) Viscosimetric Method (37). The viscometer (Brookfield DV-II+) was driven by a personal computer with Dvloader software. The spindle rotor (SC4-18) was housed in a small sample adapter filled with 5 mL of 1% chitosan in 0.1 M acetate buffer (pH 3.0, 0.02 M NaCl) and fit into a flow jacket for precise temperature control. To this was added 0.1 mL of diluted chitosanase solution (1–2 viscosimetric units) with the aid of a syringe after the desired temperature (25 °C) had been reached and just before the measurements were begun. The measurements were performed at a shear rate value of 13.2 s^{-1}. A reaction mixture with 0.1 mL of 0.1 M acetate buffer (pH 3.0, 0.02 M NaCl) instead of enzyme solution was used as a control. Viscosity was plotted against reaction time. Initial velocities (cP·min^{-1}) were graphically read from the points plotted in the subsequent 0.5 min period. At least two measurements were made and the results averaged. One unit of enzyme activity for chitosan hydrolysis was defined as the amount of enzyme decreasing 1 cP viscosity of chitosan per minute under assay conditions.

(B) Colorimetric Method. One milliliter of 0.5% chitosan in 0.1 M acetic acid, 0.9 mL of 0.1 M acetate buffer (pH 4.0), and 0.1 mL of diluted enzyme solution (0.005–0.01 colorimetric unit) in a total volume of 2 mL were incubated at 45 °C for 30 min. The reducing sugar produced was measured colorimetrically with a ferri-ferrocyanide reagent as described for the chitinase activity determination (*42*). At least two measurements were made and the results averaged. One unit of enzyme activity for glycol chitosan hydrolysis was defined as the amount of enzyme releasing 1 μmol of glucosamine per minute under assay conditions.

Chitinase Electrophoresis and Activity Staining. Sodium dodecyl sulfate–polyacrylamine gel electrophoresis (SDS-PAGE) was performed in 12.5% polyacrylamide gels (60 × 100 × 0.75 mm) containing 0.1% (w/v) glycol chitin using a vertical mini gel system (Hoefer SE-

250). Stacking gels were made of 4% (w/v) polyacrylamide (20 × 100 × 0.75 mm) (*43*, *44*). Samples (0.12 μg/μL) were boiled for 5 min with 15% (w/v) sucrose and 2% (w/v) SDS in 125 mM Tris-HCl (pH 6.8) with or without 5% (v/v) β-mercaptoethanol. Ten microliter samples were used for electrophoresis. Bromophenol blue (0.01%, w/v) was the tracking dye. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R250. Chitinase activity was detected using Calcofluor White M2R staining after glycol chitin lysis as the substrate in the gel matrix, as described by Ouakfaoui and Assein (*31*). Renaturation of enzyme activity after SDS-PAGE was in 100 mM acetate buffer (pH 5.0), containing 1% (v/v) Triton X-100 for 2 h at 37 °C with gentle shaking. Chitinase activity was detected as a dark (nonfluorecent) band against a UV fluorescent background of intact glycol chitin stained with Calcofluor White M2R.

Optimal pH Determination. The optimal pH of the chitinolytic activity of the purified chitinase/chitosanase [0.002 fluorometric unit for 4-MU-β-(GlcNAc)$_3$ hydrolysis or 0.01 colorimetric unit for glycol chitin hydrolysis] was assayed in a universal buffer (Britton and Robinson type) from pH 2 to 11 using 4-MU-β-(GlcNAc)$_3$ and glycol chitin as the substrates. The optimal pH of the chitosanolytic activity for the enzyme was assayed in a 0.1 M acetate buffer containing 20 mM NaCl from pH 2 to 6 using chitosan as the substrate with viscosimetric measurement at 25 °C at a shear rate of 13.2 s^{-1} as described for the chitosanase activity determination.

Optimal Temperature Determination. The optimal temperature of the chitinolytic activity of the purified chitinase/chitosanase was assayed at pH 4.0 from 30 to 60 °C for 4-MU-β-(GlcNAc)$_3$ hydrolysis and from 30 to 80 °C for glycol chitin hydrolysis as the substrates as described above. The optimal temperature of the chitosanolytic activity for the enzyme was assayed at pH 3 from 30 to 70 °C using chitosan as the substrate with viscosimetric measurement as described for the chitosanase activity determination.

Michaelis Constant (K_m) and Maximal Velocity (V_{max}) Determination. Michaelis constant and maximal velocity for the purified chitinase/chitosanase for glycol chitin and chitosan hydrolysis were determined as follows: 1 mL of 0.008−0.125% glycol chitin or 0.025−0.25% chitosan (in 0.1 M acetic acid), 0.9 mL of 0.1 M acetate buffer (pH 4.0), and 0.1 mL of diluted enzyme solution (0.01 colorimetric unit for glycol chitin hydrolysis) in a total volume of 2 mL were incubated at 45 °C for 30 min. The reducing sugar produced was measured colorimetrically with a ferri-ferrocyanide reagent as described for the chitinase and chitosanase assays. The maximal velocity and Michaelis constant were calculated from double-reciprocal plots according to the method of Lineweaver and Burk (*45*).

Molecular Mass Determination. The chitinase/chitosanase molecular mass was estimated using gel filtration on Superdex 75 HR using the FPLC system according to the method of Andrews (*46*). Albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa), purchased from Pharmacia Co., were used as standards. The subunit molecular mass of the enzyme was estimated using SDS-PAGE according to the procedure of Laemmli (*43*). Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa) were used as standards. The experimental conditions for SDS-PAGE were the same as described for chitinase electrophoresis and activity staining, but glycol chitin was not incorporated into the gel.

Isoelectric Point (p*I*) Determination. The p*I* of the purified chitinase/chitosanase was measured by isoelectric focusing (IEF) on PhastGel 3-9 and compared with standards from an IEF calibration kit (Pharmacia LKB) according to the manufacturer's instructions (Phast-System User's Manual). The standard IEF conditions included carrier ampholytes prefocusing at 75 Vh and sample focusing at 410 Vh at 2.5 mA and 15 °C. Samples were applied to the gel using an 8 × 1 μL comb.

Chitosan Deacetylation Degree Determination. A series of chitosans with different degrees of deacetylation were prepared by alkaline deacetylation of crab chitin as follows: For preparation of 10−20% deacetylation chitosan, 20 g of chitin was mixed with 400 mL of 40% NaOH and heated at 100 °C for 1 and 1.5 h. For preparation of 30−40% deacetylation chitosan, 20 g of chitin was mixed with 400 mL of 45% NaOH and heated at 100 °C for 1.5 and 2.5 h. For preparation of 50−70% deacetylation chitosan, 20 g of chitin was mixed with 400 mL of 50% NaOH and heated at 100 °C for 0.5, 1, and 5 h. For preparation of 90% deacetylation chitosan, 20 g of 65% deacetylation chitosan was mixed with 400 mL of 60% NaOH and heated at 100 °C for 1 h. After cooling, the supernatant was decanted out and the remaining mixture was filtered with suction through a glass filter paper. The residue was thoroughly washed with water and dried by hot air. The degree of chitosan deacetylation was measured using the colloid titration method according to the method of Toei and Kohara (*47*). A 30 mL sample of 0.5% chitosan was dissolved in 0.5% (v/v) acetic acid and titrated against 1/400 N potassium polyvinyl sulfate. A 0.1% solution of toluidine blue was used as an indicator. The end point of the titration was determined by color change of the indicator. At the end point of titration, the color of the indicator was changed from blue to pink.

Metal Ions Effect. Three-tenths of a milliliter of the purified chitinase/chitosanase (0.05 fluorometric unit) was incubated with 0.3 mL of 10 mM CuCl$_2$, CaCl$_2$, MgCl$_2$, ZnCl$_2$, NaCl, KCl, MnSO$_4$, or ethylenediaminetetraacetic acid (EDTA) at 25 °C for 30 min. The activity for 4-MU-β-(GlcNAc)$_3$ hydrolysis was then measured. The relative activity was expressed as the percentage ratio of the specific activity (units/mg) of the enzyme with metals or salts to that without metals or salts.

Chemical Modification. For determination of the effect of chemical modification of amino acid residues of the purified enzyme on enzyme activity, some amino acid side-chain modification reagents were used for modification of the enzyme. Five-tenths of a milliliter of the purified chitinase/chitosanase (11.5 μg) was incubated with 0.5 mL of the following reagents: NBS (1 mM in 50 mM acetate buffer, pH 4.0) (*48*), WRK (100 mM in 0.25 M phosphate buffer, pH 6.0) (*49*, *50*), EAM (0.5 mM in 0.2 M phosphate buffer, pH 8.0) (*51*), DEPC (5 mM in 0.25 M phosphate buffer, pH 6.0) (*52*), PHMB (1 mM in 50 mM phosphate buffer, pH 7.0) (*53*), CHD (5 mM in 0.1 M phosphate buffer, pH 8.0) (*54*), DNFB (5 mM in 0.1 M phosphate buffer, pH 8.0) (*55*), or PMSF (5 mM in 0.1 M phosphate buffer, pH 8.0) (*56*) at 30 °C for 30 min. After dialysis with 25 mM imidazole−HCl (pH 7.4), the residual enzyme activities toward 4-MU-β-(GlcNAc)$_3$ were determined. The percent inhibition is expressed as the ratio of sample activity incubated with modification reagents to the control sample's activity incubated without modification chemicals.

Thin-Layer Chromatography (TLC) of Hydrolysis Products. The purified enzyme (100 μL, 0.01 fluorometric unit) was incubated with 100 μL of 5 mM chitin or chitosan oligomers in 0.1 M acetate (pH 4.0) or 1% of chitosan or glycol chitin in 0.1 M acetate (pH 4.0) at 37 °C for 48 h. The enzymatic products were subjected to TLC with a silica gel plate 60 F$_{254}$ (Merck), in a solvent system composed of *n*-propanol/water/ammonia water (70:30:1, v/v). The TLC plates were developed by dipping them into acetone saturated with silver nitrate, followed by sprinkling with ethanol containing 0.5 N NaOH.

Protein Determination. The protein concentration (10−100 μg) was determined using the bicinchoninic acid method (*57*) using bovine serum albumin as the standard.

RESULTS

Enzyme Purification. A hydrolase with chitinase and chitosanase activity was recovered from commercial stem bromelain using SP-Sepharose ion-exchange adsorption, gel filtration on a HiLoad Superdex 75 column, ion-exchange chromatography on a HiLoad Q Sepharose FF column, and gel filtration on a Superdex 75 HR column. As shown in **Figure 1**, four protein peaks were eluted from the column, as the eluent was measured at 280 nm. An major enzyme activity peak was detected, as the eluent was assayed with 4-MU-β-(GlcNAc)$_3$ as substrate. Fractions containing the enzyme activity were collected. Most proteins without the enzyme activity were removed using this procedure. Enzyme obtained from HiLoad Superdex 75 column was further purified by ion-exchange chromatography on a HiLoad Q Sepharose FF column. As shown in **Figure 2**, a major

Table 1. Purification of Chitinase/Chitosanase from Commercial Stem Bromelain

procedure	total volume (mL)	total protein (mg)	total activity (units $\times 10^{-3}$)	specific activity (units/mg $\times 10^{-3}$)	purification (fold)	yield (%)
crude enzyme[a]	24	638	3443	5.4	1	100
SP-Sepharose Fast Flow adsorption treatment	23	376	2700	7.2	1.3	78
HiLoad Superdex 75 gel filtration	20	6.9	730	106	19.6	21
HiLoad Q Sepharose FF ion-exchange chromatography	10	1.9	220	116	21.5	6.4
Superdex 75 HR gel filtration	4	0.09	40	444	82.2	1.2

[a] One gram of commercial stem bromelain was dissolved in 25 mL of 50 mM phosphate buffer, pH 7.2. Enzyme activity was determined by using 4-MU-(GlcNAc)$_3$ as the substrate. One unit of enzyme activity was defined as the amount of enzyme releasing 1 nmol of 4-methylumbelliferone per minute under assay condition.
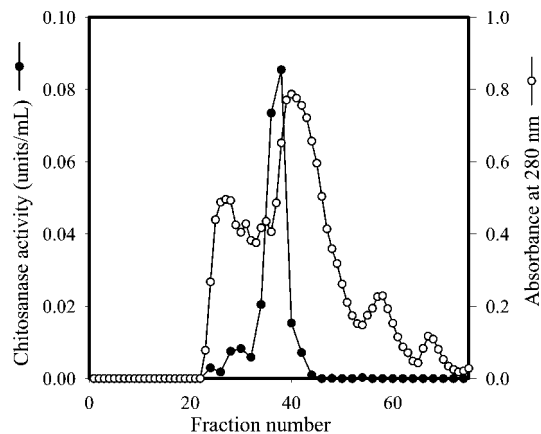

Figure 1. Column chromatography of pineapple stem chitinase/chitosanase on a HiLoad Superdex 75 column. The column was equilibrated and eluted with 25 mM imidazole–HCl buffer (pH 7.4) using an FPLC system. Fractions of 2 mL were collected.
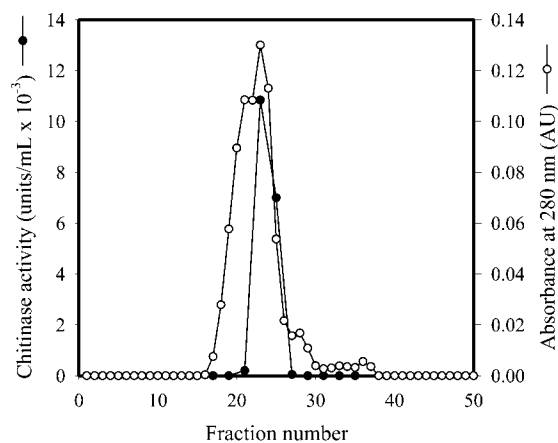

Figure 2. Column chromatography of pineapple stem chitinase/chitosanase on a HiLoad Q Sepharose FF column. The column was equilibrated with 20 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of NaCl from 0 to 0.5 M in equilibrium buffer using an FPLC system. Fractions of 1 mL were collected.

peak and three minor peaks with chitinase activity were eluted from the column. Fractions containing the major enzyme activity were collected (fractions 46−55) and further purified on a Superdex 75 HR column (**Figure 3**). The purification results are summarized in **Table 1**. Through these steps, the enzyme was purified 82.2-fold with a yield of 1.2%. At the final stage of purification, the recovery of the enzyme activity was rather low. This may be due to the removal of some minor activity peaks during the HiLoad Q Sepharose FF ion-exchange chromatography. It is also possible that some of the activity loss occurred during dialysis and lyophilization steps.

Figure 3. Column chromatography of pineapple stem chitinase/chitosanase on a Superdex 75 HR column. The column was equilibrated with 25 mM imidazole–HCl buffer (pH 7.4, 0.1 M NaCl) and eluted with 25 mM Tris-HCl buffer (pH 7.4, 0.1 M NaCl) using an FPLC system. Fractions of 0.5 mL were collected.
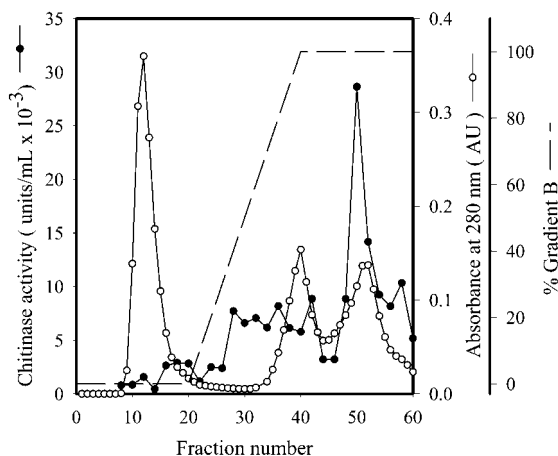
SDS-PAGE and Enzyme Activity Staining. When the purified enzyme was denatured with SDS without a reducing agent, the purified enzyme exhibited a single protein band at 29.5 kDa (**Figure 4A**, lane 1), as examined by SDS-PAGE. The enzyme also showed an activity band for glycol chitin hydrolysis (**Figure 4B**, lane 3), as examined by SDS-PAGE and activity staining. The activity band matched the corresponding protein band. When the enzyme was denatured with SDS and a reducing agent, the enzyme exhibited a single protein band at 15.8 kDa (**Figure 4A**, lane 2), as examined by SDS-PAGE. However, the enzyme did not exhibit activity for glycol chitin hydrolysis (**Figure 4B**, lane 4), as examined by SDS-PAGE and activity staining. These results indicate that the enzyme denatured in the presence of SDS alone could be renatured after SDS-PAGE. However, disulfide bond reduction of this enzyme by a reducing agent renders irreversible denaturation in this enzyme.

pH Effect on Enzyme Activity. The purified chitinase/chitosanase had an optimal pH of 4 for 4-MU-β-(GlcNAc)$_3$ and glycol chitin hydrolysis. However, the enzyme had an optimal pH of 3 for chitosan hydrolysis. Thus, the purified enzyme revealed both chitine and chitosane lytic activities. In addition, it also showed chitotriosidase activity. Four acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissues had an optimal pH of 5 for chitosan hydrolysis and an optimal pH between 3.5 and 5.5 for colloidal chitin hydrolysis (*23*).

Temperature Effect on Enzyme Activity. The purified chitinase/chitosanase had an optimal temperature of 50 °C for 4-MU-β-(GlcNAc)$_3$ and chitosan hydrolysis. However, the enzyme had an optimal temperature of 60 °C for glycol chitin hydrolysis.

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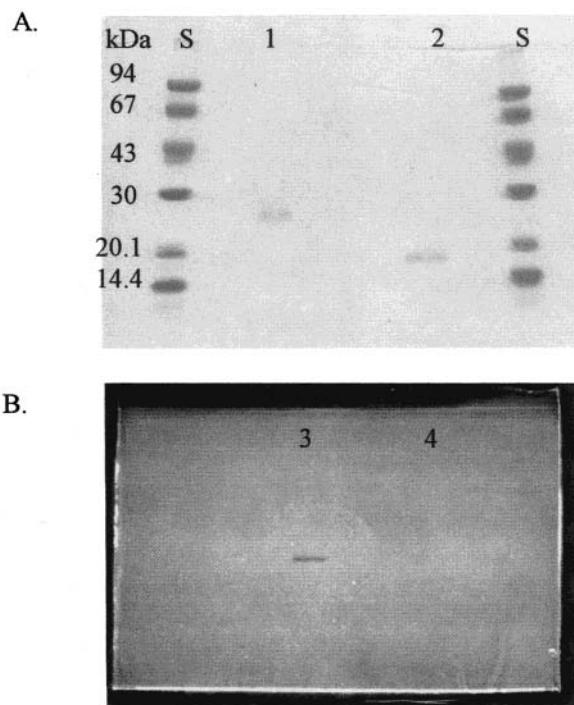
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A.

B.

Figure 4. SDS-PAGE of pineapple stem chitinase/chitosanase and activity staining for glycol chitin hydrolysis: (A) protein staining [(lane S) protein molecular weight markers; (lane 1) enzyme denatured in the presence of SDS alone; (lane 2) enzyme denatured in the presence of SDS plus 2-mercaptoethanol]; (B) activity staining [(lane 3) enzyme denatured without reducing agent and renatured in 100 mM acetate buffer (pH 5.0, 0.1% Triton X-100)]. Chitinase activity was detected by Calcofluor White M2R staining after lysis of glycol chitin as substrate in the gel matrix.

Table 2. Kinetic Constants of Pineapple Stem Chitinase/Chitosanase

substrate	V_{max} (μmol of GlcNAc or GlcN/min/mg)	K_m (mg/mL)	V_{max}/K_m (μmol of GlcNAc or GlcN/min/mg·mL/mg)
glycol chitin	2.67	0.20	13.35
chitosan	3.50	0.88	3.98

Michaelis Constant and Maximal Velocity. The substrate concentration effect on the hydrolase rate for glycol chitin and chitosan hydrolysis was determined. The Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from Lineweaver–Burk plots. (*45*) For glycol chitin hydrolysis, the enzyme had a K_m of 0.20 mg/mL and a V_{max} of 2.67 μmol of GlcNAc/min/mg. For chitosan hydrolysis, the enzyme had a K_m of 0.88 mg/mL and a V_{max} of 3.50 μmol of GlcN/min/mg (**Table 2**). These results indicate that the enzyme had a higher lytic rate constant (V_{max}/K_m) for glycol chitin (13.35 μmol of GlcNAc/min/mg·mL/mg) hydrolysis than for chitosan hydrolysis (3.98 μmol of GlcN/min/mg·mL/mg) at substrate concentrations much lower than K_m. However, the enzyme had a higher lytic rate toward chitosan (V_{max} = 3.50 μmol of GlcN/min/mg) than toward glycol chitin (V_{max} = 2.67 μmol of GlcNAc/min/mg) at substrate concentrations much higher than K_m.

Molecular Mass. The molecular mass of the enzyme was 31.4 kDa, as estimated by gel filtration on a Superdex 75 HR using the FPLC system. This value was close to the value estimated by SDS-PAGE under nonreducing conditions (29.5 kDa; **Figure 4A**, lane 1). However, this value is also twice the value estimated by SDS-PAGE under reducing conditions (15.8
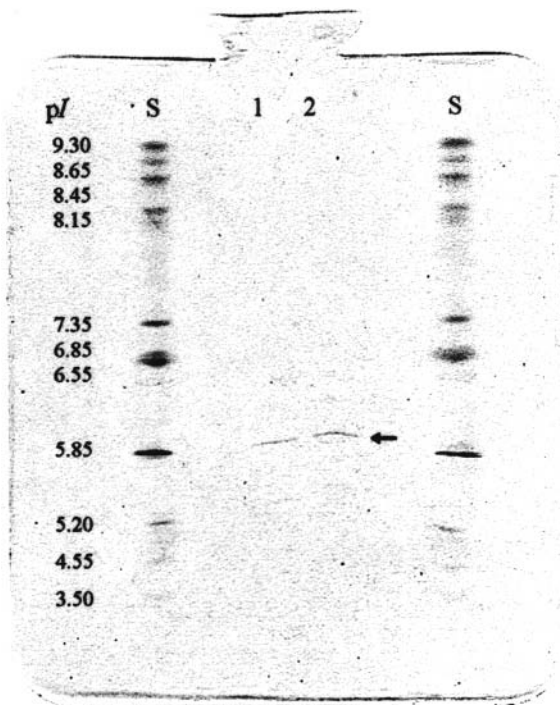

Figure 5. IEF electrophoresis of pineapple stem chitinase/chitosanase: (lanes 1 and 2) purified chitinase/chitosanase; (lane S) standard proteins [trypsinogen (pI = 9.30); lentil lectin, basic band (pI = 8.65); lentil lectin, middle band (pI = 8.45); lentil lectin, acidic band (pI = 8.15); myoglobin, basic band (pI = 7.35); myoglobin, acidic band (pI = 6.85); human carbonic anhydrase β (pI = 6.55); bovine carbonic anhydrase β (pI = 5.85); β-lactoglobulin A (pI = 5.20); soybean trypsin inhibitor (pI = 4.55); amyloglucosidase (pI = 3.50)]. IEF was performed on a PhastGel IEF 3-9 according to the PhastSystem user's manual.

kDa; **Figure 4A**, lane 2). Therefore, the enzyme probably consisted of two polypeptide chains with disulfide bonds.

Isoelectric Point. The isoelectric point (pI) of the purified enzyme was 5.9, as estimated by IEF electrophoresis (**Figure 5**). Thus, it is an acidic hydrolase.

Chitosan Deacetylation Effect on Enzyme Activity. The chitosan deacetylation degree affected the activity of the enzyme. The chitosan polymers with 10–90% deacetylation were susceptible to the enzyme action. The most susceptible to hydrolysis were 30–80% deacetylation (**Figure 6**). Chitosan deacetylated <30% or >80% became less susceptible.

Effect of Metal Ions, Salts, and Chemical Modification Reagent on Enzyme Activity. As shown in **Table 3**, the enzyme activity for 4-MU-β-(GlcNAc)$_3$ hydrolysis was not affected by K$^+$, Na$^+$, Cu^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, and EDTA, but it was inhibited by Hg^{2+} and Ag$^+$. If an amino acid side chain involved in the catalytic activity is chemically modified, the enzyme will be inactivated. A number of reagents were used to modify the purified enzyme. Among the chemical modification reagents tested, the enzyme activity was not affected by WRK, EAM, CHD, DNFB, PMSF, and DEPC, but it was inhibited by NBS and PHMB (**Table 4**). Hg^{2+} and Ag$^+$ heavy metal ions and PHMB are mercaptide-forming agents. They can react with enzymes containing SH groups and subsequently inhibit their activity. NBS is a protein-oxidizing agent. The oxidizing reaction is usually specific for tryptophan and the SH group. However, the enzyme activity was not completely inhibited by PHMB and NBS. Cysteine may be involved only in holding the monomers together, and activity could be destroyed when the dimer structure is disrupted.
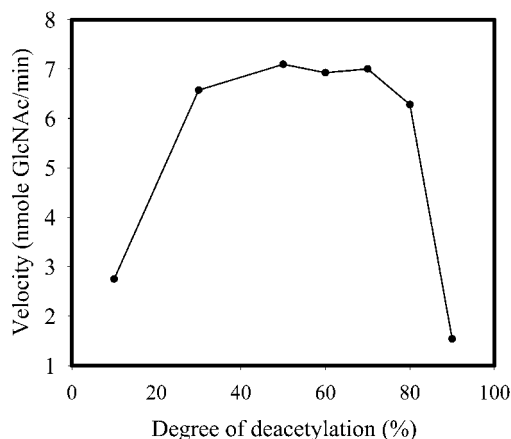
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Figure 6. Effect of degree of deacetylation of chitosan on the activity of pineapple stem chitinase/chitosanase. Chitosanase activity was assayed with 0.25% chitosan in 0.1 M acetic acid (pH 4.0) at 45 °C. The reducing sugars produced were measured colorimetrically with a ferri-ferrocyanide reagent.

Table 3. Effect of Various Metal Ions and Salts on the Activity of Pineapple Stem Chitinase/Chitosanase

reagent	concentration (mM)	relative activity (%)
none		100
$HgCl_2$	0.25	49.7
$AgNO_3$	0.25	66.3
KCl	5	93.9
NaCl	5	93.9
$CuCl_2$	5	89.6
$CaCl_2$	5	96.1
$MgCl_2$	5	96.4
$ZnCl_2$	5	94.6
EDTA	5	107.3

Table 4. Effect of Various Chemical Modification Agents on the Activity of Pineapple Stem Chitinase/Chitosanase

reagent[a]	concentration (mM)	relative activity (%)
none		100
WRK	50	94
NBS	0.5	52
EAM	0.25	114
CHD	2.5	109
DNFB	2.5	98
PMSF	2.5	116
DEPC	2.5	111
PHMB	1	58

[a] WRK, Woodward's reagent K; NBS, *N*-bromosuccinimide; EAM, ethyl acetimidate; CHD, 1,2-cyclohexanedione; DNFB, 2,4-dinitro-1-fluorobenzene; PMSF, phenylmethanesulfonyl fluoride; DEPC, diethyl pyrocarbonate; PHMB, *p*-hydroxymercuribenzoic acid.

Chitosan, Glycol Chitin, and Chitosan Oligosaccharides Hydrolysis Products. As shown in **Figure 7**, standard chitosan oligosaccharides (glucosamine oligomers) with chain lengths from 1 to 6 were not hydrolyzed by the enzyme (**Figure 7**, lanes 1−6). The products of chitosan hydrolysis were glucosamine, chitobiose, and oligomers of glucosamine with a chain length longer than 6 (**Figure 7**, lane 7). The products of glycol chitin hydrolysis were dimer, trimer, and tetramer of *N*-acetylglucosamine (**Figure 7**, lane 8), as compared with the relative mobility of the standard chitin oligomers as shown in **Figure 8**.

Chitin Oligosaccharides Hydrolysis Products. As shown in **Figure 8**, standard chitin oligosaccharides (oligomers of
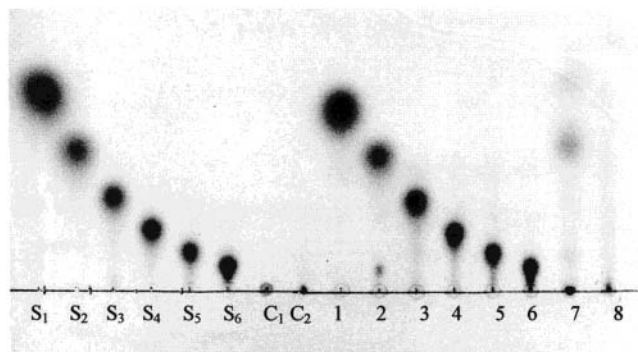

Figure 7. Thin-layer chromatogram showing hydrolysis of glucosamine (GlcN) oligomers, chitosan, and glycol chitin using pineapple stem chitinase/chitosanase. Lanes S_1–S_6 denote standard glucosamine oligomers GlcN, $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$, and $(GlcN)_6$, respectively. Lanes C_1 and C_2 denote chitosan (82% deacetylation) and glycol chitin. Lanes 1–6 denote hydrolysis products of glucosamine oligomers from monomer to hexamer, respectively. Lanes 7 and 8 denote hydrolysis products of chitosan and glycol chitin, respectively.
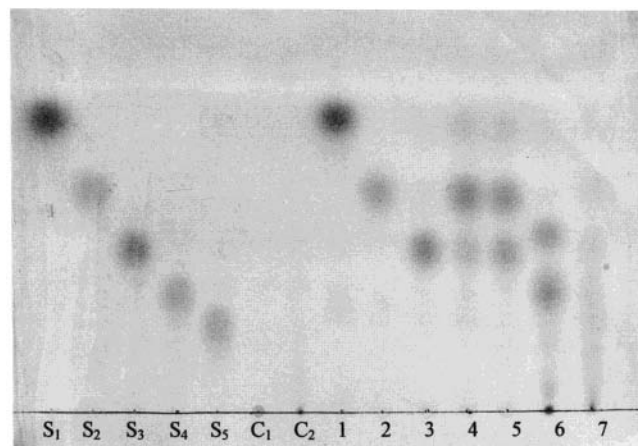

Figure 8. Thin-layer chromatogram showing hydrolysis of *N*-acetylglucosamine (GlcNAc) oligomers, glycol chitin, and chitosan using pineapple stem chitinase/chitosanase. Lanes S_1–S_5 denote standard *N*-acetylglucosamine oligomers GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$, respectively. Lanes C_1 and C_2 denote chitosan (82% deacetylation) and glycol chitin. Lanes 1–5 denote hydrolysis products of *N*-acetylglucosamine oligomers from monomer to pentamer, respectively. Lanes 6 and 7 denote hydrolysis products from chitosan and glycol chitin.

N-acetylglucosamine) with chain lengths from 1 to 3 were not hydrolyzed by the enzyme (**Figure 8**, lanes 1−3). However, tetramer and pentamer of *N*-acetylglucosamine were hydrolyzed into monomer, dimer, and trimer (**Figure 8**, lanes 4 and 5). The tetramer should be the shortest oligomer still recognized as a substrate by this enzyme. The products of chitosan and glycol chitin hydrolysis (**Figure 8**, lanes 6 and 7) were the same as shown in **Figure 8** (lanes 7 and 8).

DISCUSSION

This paper described the purification and characterization of a hydrolase with chitinase and chitosanase activity from commercial stem bromelain preparation. The isoelectric point of the enzyme was 5.9, as determined by IEF electrophoresis. The molecular mass of the enzyme was calculated as ∼30 kDa by both gel filtration and SDS-PAGE without a reducing agent. However, the enzyme had a molecular mass of 15.8 kDa by SDS-PAGE with a reducing agent, indicating that the enzyme

consisted of two polypeptide chains with disulfide bonds. A hydrolase with dual chitinase and chitosanase activities was described by Pegg and Young (*58*) in tomato stems; it had an isoelectric point of 8.0 and a molecular mass of 27 kDa by gel filtration and of 31 kDa by SDS-PAGE. Four acidic hydrolases with chitinase and chitosanase activities were identified and characterized by Osswald et al. (*23*) from sweet orange callus tissue. These citrus chitinase/chitosanases had isoelectric points from pH 5.29 to 5.4 and molecular masses between 29.1 and 30.5 kDa. Grenier and Asselin (*24*) reported chitosanase in stressed barley, cucumbers, and tomatoes that degraded only chitosan. Ouakfaoui and Asselin (*30, 31*) found acidic and basic chitosanase isoforms with molecular masses between 10 and 23 kDa in the roots, leaves, fruits, and flowers of dicots and monocots using activity-staining techniques on gels with glycol chitosan as the substrate. The hydrolase we purified from a stem bromelain preparation degraded both glycol chitin and chitosan. Analysis of the reaction products from glycol chitin and chitosan with 82% deacetylation indicated that the major products were dimer and trimer oligomers and some oligomers with a longer chain length. However, a minor amount of monosaccharide was also present. These results indicate that the enzyme is endohydrolase. The chitosan polymers most susceptible to hydrolysis by this enzyme were 30–80% deacetylated. Chitosans with a degree of deacetylation below 30% or higher than 80% were less susceptible to the enzyme action. Thus, both glucosamine and *N*-acetylglucosamine are necessary for optimal activity. Citrus chitinase/chitosanases were active to 80–100% deacetylated chitosan, and the enzyme activity decreased as the degree of deacetylation of chitosan increased (*23*). Chitosanases from *Myxobacter* AL-1 (*59*), *Streptomyces griseus* (*60*), and *Bacillus* no. 7-M (*61*) were reported to be active toward chitin or CM-cellulose. Up to now, little is known about plant chitosanase or chitinase with chitosanase activity, although plant chitinases have been extensively studied (*11*). The hydrolase we purified showed activities toward *N*-acetylchitotrioside, glycol chitin, and chitosan. For glycol chitin hydrolysis, the enzyme had a K_m of 0.20 mg/mL. This value was less than the K_m of other plant chitinase (0.3–0.9 mg/mL) (*11*). The enzyme also showed a higher lytic rate constant (V_{max}/K_m) for glycolchitin hydrolysis than for chitosan hydrolysis under circumstances of low substrate concentration. However, at substrate concentrations much higher than K_m, the enzyme had a higher lytic rate toward chitosan than toward glycol chitin. Chitinase with chitosanase activity (or vice versa) would be more effective against a wider variety of fungal pathogens and could generate oligosaccharide elicitors from chitin or chitosan.

Chitosan degradation products have various potential applications (*10–15*). The development of viable processes for the hydrolysis of chitosan is attracting growing interest (*62*). Crude stem bromelain displayed lytic activity toward chitosan and water-soluble chitin (*36*) and is a potential inexpensive enzyme for chitosan hydrolysis. This research demonstrated that the hydrolysis of chitin and chitosan using stem bromelain was achieved by a hydrolase with chitinase and chitosanase activity present in the crude enzyme preparation and not by the bromelain itself.

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